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The effect of nutritional condition by two nucleic acid derived indices on the growth to post-flexion of Atlantic bluefin tuna and Atlantic bonito larvae

Edurne Blanco[a,*], Patricia Reglero[a], Alma Hernández de Rojas[b], Aurelio Ortega[c], Fernando de la Gándara[c], Arild Folkvord[d,e]

[a] Instituto Español de Oceanografía, Centre Oceanogràfic de les Balears, Ecosystem Oceanography Group (GRECO), Moll de Ponent s/n, 07015 Palma de Mallorca, Spain
[b] Instituto Español de Oceanografía, Centro Oceanográfico de Gijón, 33212 Gijón, Spain
[c] Instituto Español de Oceanografía, Centro Oceanográfico de Murcia, 30860 Puerto de Mazarrón, Murcia, Spain
[d] Department of Biological Sciences, University of Bergen, 5020 Bergen, Norway
[e] Institute of Marine Research, 5817 Bergen, Norway

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ABSTRACT

Notochord flexion increases the swimming capacity of fish larvae, aids in the capture of mobile prey, and coincides with the timing of when the physiological capacities of larvae begin to develop significantly, allowing an early shift to piscivory. Therefore, reaching the flexion stage as soon as possible can be considered beneficial for the growth and survival of the larvae. Individual growth differences of larvae from the same cohort are very common before reaching flexion and the potential explanation is still unknown. In this study, we examined if the nutritional status of the larvae, measured by the RNA:DNA and DNA:DW ratios, explains the differences in the development of notochord flexion in laboratory-reared Atlantic bluefin tuna (ABFT; *Thunnus thynnus*) and Atlantic bonito (AB; *Sarda sarda*) larvae. Moreover, we described the ontogeny of the different stages and condition indices. The daily average condition of ABFT estimated by the RNA:DNA and DNA:DW ratios increased with larval age and developmental stage, whereas in AB it was not. In both species, bigger larvae at a specific age had higher nutritional condition than smaller larvae regardless of developmental stage. However, in ABFT the DNA:DW ratio indicated lower condition for the biggest larvae within developmental stages F0 (pre-flexion) and F3 (post-flexion). After removing the size effect, larger individuals of ABFT within flexion stages F1 and F2 had higher condition than any other larval stages, whereas in AB an indistinct trend within stages was found. Our results confirm that in ABFT the RNA:DNA ratio is correlated with faster development while the DNA:DW ratio is negatively correlated with the time to flexion. The trends found in the DNA:DW ratio points at cell growth mechanisms being a better indicator of larval condition that the RNA:DNA ratio. Our findings suggest that under culture, *ad libitum* conditions, larval nutritional conditions measured by nucleic acid ratios, might not be enough to explain developmental differences in fast growing species.

1. Introduction

The nutritional condition of marine fish reflects the energy reserves available for maintenance, growth and activity, and the individual responses to variability in food supply and feeding success are ultimately related to survival (Anderson, 1988; Cushing, 1990; Hjort, 1914). During their first weeks of life —a period characterized by high mortality rates (Bailey and Houde, 1989; Leggett and Deblois, 1994)— and after they have consumed all the lipids of the yolk, fish larvae have low energy reserves, and their tissues and organs are under progressive and intense differentiation and development (Fuiman, 1983; Kendall et al., 1984; Osse and Van den Boogaart, 1995; Pittman et al., 2013).

Suboptimal feeding may lead to the death of larvae directly, by starvation, or indirectly, through prolonged stage duration and higher vulnerability to predation (Fiksen and Jørgensen, 2011; Folkvord et al., 2015). Differences in nutritional condition can also generate growth variability, and larvae of the same age may show individual differences, consequently influencing their success during this critical early life stage (Takebe et al., 2012; Tanaka et al., 2010).

One important event in the early life of fish is the flexion of the notochord, which accompanies the hypocordal development of the homocercal caudal fin during the larval stage (Kendall et al., 1984). This physiologically stressful moment is associated with changes in body shape, resulting in improvements in swimming capacity and

* Corresponding author.
E-mail address: edurne.blanco.rod@gmail.com (E. Blanco).

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feeding techniques (Kendall et al., 1984; Osse and Van den Boogaart, 1995). It is also considered an inflexion point in the early stages of the life cycle of a fish, where growth and survival rates increase due to enhanced access to a wider spectrum of prey and increased predation avoidance (Kaji, 2003; McFarlane et al., 2000; Somarakis and Nikolioudakis, 2010).

Atlantic bluefin tuna (ABFT; *Thunnus thynnus*), and Atlantic bonito (AB; *Sarda sarda*), are the only scombrid species in the Mediterranean Sea that have been successfully reared in captivity (De la Gándara et al., 2012; Ortega, 2015). Experimental studies have shown that these two species become piscivorous already during the larval stage, once the flexion of the notochord has occurred (Blanco et al., 2017; Reglero et al., 2014). This change in diet increases survival and is required for sustaining growth and the high metabolic requirements of these species (Reglero et al., 2014). The flexion coincides with the start of the development of an adult-type digestive system, with blind sac, gastric glands, pyloric caeca and digestive enzymes that allow for the digestion of fish prey (Kaji, 2003; Miyashita et al., 1998; Yúfera et al., 2014). On the other hand, the completion of the flexion is externally accompanied by the development of the fin rays and the development of the caudal muscle fiber, which will allow for improved swimming (Kendall et al., 1984; Osse and Van den Boogaart, 1995; Roy et al., 2014). In general, bluefin tunas are also characterized by having a very low tolerance to starvation, with up to 50% of the larvae dying one day after starved conditions and showing an immediate growth retardation. Therefore, those with low feeding incidence (or low nutritional condition) will rapidly die (e.g. Pacific bluefin tuna, Tanaka et al., 2008). Growth differences in individual larvae from the same cohort have been studied in Pacific bluefin tuna before reaching the flexion stage using otolith back-calculation analyses in laboratory-reared and field-captured larvae (Takebe et al., 2012; Tanaka et al., 2006). However, the effect of the nutritional condition in the larval growth variability of tuna has only been studied in two laboratory studies using stable isotope analyses in Pacific bluefin tuna larvae offered different preys (Tanaka et al., 2010, 2014).

Inter-individual condition differences during the earliest life stages, have been largely overlooked before now. The collection of post-flexion larvae in the field is a difficult task, mainly due to their ability to evade sampling nets once their swimming capacity has improved (Satoh et al., 2008). Additionally, assessing individual nutritional condition from field samples is difficult, as extrinsic factors experienced by larval fish are often changing, and different larvae of the same cohort might experience different environmental conditions in a single day (Peck et al., 2012). Food availability and water temperature are the main factors affecting the nutritional condition of fish larvae (e.g. Buckley et al., 1984; Clemmesen, 1994; Foley et al., 2016; Folkvord et al., 1996). Laboratory studies allow us to obtain larval sizes rarely captured in the field and to identify individual responses in the nutritional condition to different feeding and thermal controlled conditions.

Larval nutrition has been suggested as a possible cause of the high mortality in laboratory-reared ABFT and AB (Reglero et al., 2014) but this has never been tested. To date, lipid content and histology of the organs have been used as a proxy of nutritional condition in ABFT (Ortega and Mourente, 2010; Yúfera et al., 2014). For Pacific bluefin tuna, the ontogenetic changes in nutritional condition have been analyzed in the laboratory and in the field (Tanaka et al., 2007, 2008), whereas data for ABFT is only available for field-captured larvae (García et al., 2006). Nutritional condition is associated with the food supply and feeding success of the fish and therefore variability in the trophic environment is reflected in nutritional condition.

In this study, we examined if the nutritional status of laboratory reared ABFT and AB larvae could explain individual differences in the timing of notochord flexion. We use two different nucleic acid derived indices to determine larval fish nutritional condition: the RNA:DNA and the DNA:dry weight (DNA:DW) ratios. The RNA:DNA ratio is an index of cell metabolic intensity and it is used as an approach for recent growth and recent nutritional condition of fish larvae (Clemmesen, 1994; Folkvord et al., 1996). The amount of DNA is stable under changing environmental situations, reflecting the number of cells of an individual, whereas the amount of RNA is directly proportional to the protein synthesis capacity in the cell. The RNA is highly dependent on food quantity and varies with age, life stage, organism size, disease state, changing environmental conditions and diel differences (Buckley et al., 1999; Rooker and Holt, 1996). During food deprivation, the nutritional condition, and therefore the RNA:DNA ratio, decreases, reflecting the cessation of protein synthesis and somatic growth (e.g. Clemmesen, 1994). Therefore, well-fed larvae are metabolically more active, grow faster and have relatively higher RNA:DNA ratios compared to poor-fed larvae with less active metabolism (Clemmesen, 1987, 1994). The RNA:DNA ratio is sensitive to changes in specific growth rates, both in terms of length and weight, and provides information on the feeding environment of the larvae within a time frame of days prior to sampling. This can be interpreted as recent growth capacity and can be useful to examine the survival processes (Bergeron, 1997; Rooker and Holt, 1996). On the other hand, the DNA:DW ratio indicates the cell condition, since cell weight decreases while the amount of DNA remains constant during a reduction in the nutritional condition of the larvae (Bergeron, 1997). Opposite, the DNA:DW ratio increases when nutritional condition decreases, since more cells are present for the same weight of tissue of starved larvae (Bergeron, 1997; Chícharo and Chícharo, 2008). Some authors consider being the DNA:DW ratio a more stable and sensitive ratio during the early stages than the RNA:DNA ratio due to the high variability of RNA content during the larval stage (Bergeron, 1997).

In this study we sought to determine the effect of the nutritional condition on larval growth and developmental variability to post-flexion stage and to describe the ontogeny of the different stages and condition indices in ABFT and AB larvae. Two nucleic acid derived indices, RNA:DNA and DNA:DW ratios, were used as condition measures to test the hypotheses that 1) at a specific day, larvae with higher nutritional condition were bigger in size at any developmental stage than the smaller larvae with lower nutritional condition and 2) at a specific size, more developed larvae had higher nutritional condition than the less developed ones.

2. Material and methods

2.1. Atlantic bluefin tuna experiment

Fertilized eggs of ABFT were collected from naturally-spawning captive adults in the farming facilities at El Gorguel, Cartagena (SE Spain), owned by Caladeros del Mediterráneo S.L. In the laboratory, floating and sinking eggs (at natural seawater salinity of 37) were separated in a 5 L bucket. Floating ABFT eggs were incubated in 400 L tanks at 28 °C under a continuous light regime. A few hours after incubation, 60,000 ABFT eggs were transferred to four 1500 L cylindrical tanks, with 15,000 eggs per tank and the sea water temperature set at 28 °C. Assuming a hatching rate of 85–90% (personal observation), initial larval density ranged between 8.5 and 9 larvae L^{-1}. Water temperature in the incubators and in the rearing tanks was controlled using heaters—isolated to avoid larval mortality—inside the tanks. The water temperature was measured continuously by a HOBO data logger (www.onsetcomp.com). Rearing was conducted with a photoperiod of 14L:10D, similar to natural conditions in the area.

Cultivated microalgae of *Nannochloropsis gaditana* were added twice each day from 0 to 3 days post hatch (dph). Afterwards, a paste of concentrated *Chlorella* (Super fresh Chlorella SV-12, Chlorella Industry Co., Ltd., Japan) was added three times per day in each tank. The larvae were fed following the technique described in De la Gándara et al. (2012). Enriched rotifers, *Brachionus plicatilis*, were added from 3 dph at a concentration of 5 rot mL^{-1} to guarantee *ad libitum* conditions. From 14 dph onwards, gilthead sea bream (*Sparus aurata*) yolk-sac larvae of

Table 1
Summary of the differences between Atlantic bluefin tuna and Atlantic bonito experiments. Daily total number of larvae sampled (n) in each tank replicate (R1, R2, R3 and R4) per larval day post hatch (dph). Average temperature in °C during the corresponding days and the average daily standard length (SL, mm) and dry weight (DW, mg) of the sampled larvae is shown in the table. In Atlantic bluefin tuna at 20 dph, the standard length is shown as a range due to the high size variability. Variability around the mean is displayed as standard deviation (± SD).

Species	Dph	Sampling (n)				SL (mm)	DW (mg)
		R1	R2	R3	R4		
Atlantic bluefin tuna 27.7 ± 0.4 °C	8	40	40	40	40	5.1 ± 0.5	0.13 ± 0.05
	10	40	40	40	40	6.2 ± 0.7	0.32 ± 0.15
	11	40	40	40	40	6.7 ± 0.6	0.42 ± 0.18
	12	40	40	40	40	7.0 ± 0.7	0.58 ± 0.26
	13	40	40	40	40	7.5 ± 0.6	0.77 ± 0.26
	20	40	40	40	0	11.3–24.6	4.46–50.1
Atlantic bonito 26.2 ± 0.9 °C	5	20	20	20	0	5.7 ± 0.3	0.16 ± 0.03
	6	30	30	30	0	6.6 ± 0.5	0.28 ± 0.06
	7	20	20	20	0	7.3 ± 0.4	0.45 ± 0.09
	8	20	20	20	0	8.0 ± 0.3	0.68 ± 0.13
	9	All	All	All	0	8.2 ± 0.3	0.77 ± 0.13

0–2 dph (3.4 ± 0.04 mm) were added, providing up to 300 preys per individual twice daily. Pseudo–green water technique was used during the entire rearing period to avoid the depletion of the nutritional condition of the rotifers and the resulting effect on larval development (Yamamoto et al., 2009).

Several small samplings were carried out every day before 8 dph in ABFT to follow the development of the larvae and to accurately identify the first day any larvae started to flexion. Experimental samplings started as soon as we found flexion stage larvae and finished when at least 50% of the larvae were found to be in the post-flexion stage to minimize the possibility that cannibalism might result in differential feeding. The experiment lasted for 20 days, from 0 to 20 dph. 40 larvae per tank were randomly sampled at 8, 10, 11, 12 and 13 dph. At 13 dph, all the remaining ABFT larvae in the tanks were counted to estimate survival and a total of 1845 larvae were transferred to three new 1500 L cylindrical tanks, with up to 615 larvae randomly distributed in each tank. Hereafter, the larvae were cultivated until they were 20 dph and reached the early juvenile stage, at which point 40 larvae per tank were sampled and all the remaining larvae were counted for survival estimates. Larval sampling was carried out every day at the same time, early in the morning and in darkness using a long siphon whose diameter was increased according to larval sizes. By using the siphon, we ensured the sampling of the weaker (probably in the upper part, easy to catch) and stronger (probably in the bottom, hard to catch) larvae.

2.2. Atlantic bonito experiment

Fertilized eggs of AB were obtained from stripped spawning adult individuals collected in an almadraba trap in La Azohía (Murcia, SE Spain). In the laboratory, floating and sinking eggs (at natural seawater salinity of 37) were separated in a 5 L bucket. Floating AB eggs were incubated in 400 L tanks at 26 °C under a continuous light regime. Just after hatching, at 0 dph, 11,250 AB larvae were transferred to three 1500 L cylindrical tanks, with 3750 larvae in each tank, 2.5 larvae L^{-1}, and the water temperature set at 26 °C. Water temperature in the incubators and in the rearing tanks was controlled using heaters—isolated to avoid larval mortality—inside the tanks. The water temperature in each tank was measured continuously by a HOBO data logger (www.onsetcomp.com). Rearing was conducted with a photoperiod of 14L:10D, similar to natural conditions in the area.

Cultivated microalgae of *Nannochloropsis gaditana* were added twice each day, from 0 to 2 dph. Afterwards, a paste of concentrated *Chlorella* (Super fresh Chlorella SV-12, Chlorella Industry Co., Ltd., Japan) was added three times per day in each tank. The larvae were fed following the technique described in De la Gándara et al. (2012). Enriched rotifers, *Brachionus plicatilis*, were added from 2 dph at a concentration of 5 rot mL^{-1} to guarantee *ad libitum* conditions. Pseudo-green water technique was used during the entire rearing period to avoid depletion of the nutritional condition of the rotifers and the resulting effect on larval development (Yamamoto et al., 2009).

Several small samplings were carried out every day before 5 dph to follow the development of the larvae. Experimental samplings started as soon as we found flexion stage larvae and finished when at least 50% of the larvae were found to be in the post-flexion stage, to avoid the possible cannibalism of those that first reached the post-flexion stage. The experiment lasted for 9 days, from 0 to 9 dph. 20 larvae were randomly sampled in each tank at 5, 7 and 8 dph while 30 larvae per tank were sampled at 6 dph (Table 1). The last day, 9 dph, all the remaining larvae in the tanks were counted and sampled. Larval sampling was carried out every day at the same time, early in the morning and in darkness.

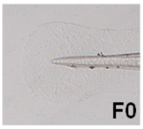
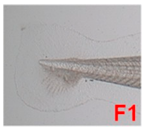
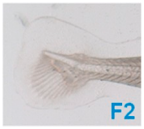
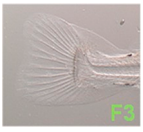
2.3. Laboratory analyses

Immediately after sampling, the larvae were anesthetized using clove oil (Guinama© Spain), individually photographed using an image analysis system connected to a microscope (Leica Microsystem, Inc., Bannockburn, IL) and individually frozen in vials at −80 °C. ABFT larvae were submerged in RNAlater® before preserving at −80 °C. Later, in the laboratory, the larvae conserved in RNAlater® (Sigma-Aldrich R0901) were rinsed with milliQ water and lyophilized to estimate individual dry weight (DW) (to the nearest 0.01 mg) and larval nutritional condition. The standard length (SL) of the sampled fish was measured to the nearest 0.1 mm from the anterior margin of the snout to the posterior margin of the hypural plate of the notochord. Four different developmental phases based on morphological characteristics of the notochord and caudal fin were determined following a modified version of the criteria of De la Gándara et al. (2013), a modified version for *Thunnus thynnus* of Kendall et al. (1984) and Kaji et al. (1996) (Table 2): 1) larvae in pre-flexion (F0), 2) larvae with development of the first caudal fin rays (F1), 3) larvae in flexion (F2) and 4) larvae in post-flexion (F3).

2.4. Nutritional condition: nucleic acid analyses

RNA:DW and DNA:DW ratios were determined using a modification of the method described by ICES (2004). RNA:DW and DNA:DW were individually measured using the whole larval body and all the reagents

Table 2
Description of the four different developmental stages used in this article, based on morphological characteristics of the notochord and caudal fin rays.

Stage	Nomenclature	Description	Example
Pre-flexion	F0	Straight notochord	
First caudal fin rays	F1	Straight notochord with some rays in the ventral side	
Flexion	F2	Bending upward of the notochord tip in a very clear angle with an increase in the amount of fin rays	
Post-flexion	F3	The final tip of the notochord disappears. Definition of the hypural plate and caudal fork. The posterior margin of the upper hypural plate is at 90° from the notochord axis	

were prepared using Tris-EDTA buffer (0.05 M TRIS, 0.1 M NaCl, 0.01 M EDTA, adjusted to pH 8.0 with HCl). First, lyophilized larvae were rehydrated by transferring to a mixture of Tris-EDTA buffer and sodium dodecyl sulfate 0.7% (SDS) for 15 min at 4 °C. Once rehydrated in the vial, the larvae were completely disintegrated by applying two 10-s ultrasound pulses (Bandelin Sonoplus). An increase in the temperature of the homogenates was avoided by keeping all the vials on ice. The homogenate was centrifuged at 3800 × *g* during 8 min at 4 °C. Two supernatant aliquots were taken, one for the measurements of the total nucleic acids (RNA + DNA) and another one for the measurement of the DNA content (μg fish^{-1}). The DNA measurement was carried out by incubating the samples with RNase A (type I-AS, Sigma-Aldrich) at 37 °C during 30 min. The difference between the total nucleic acids fluorescence and the DNA fluorescence was corrected to determine the

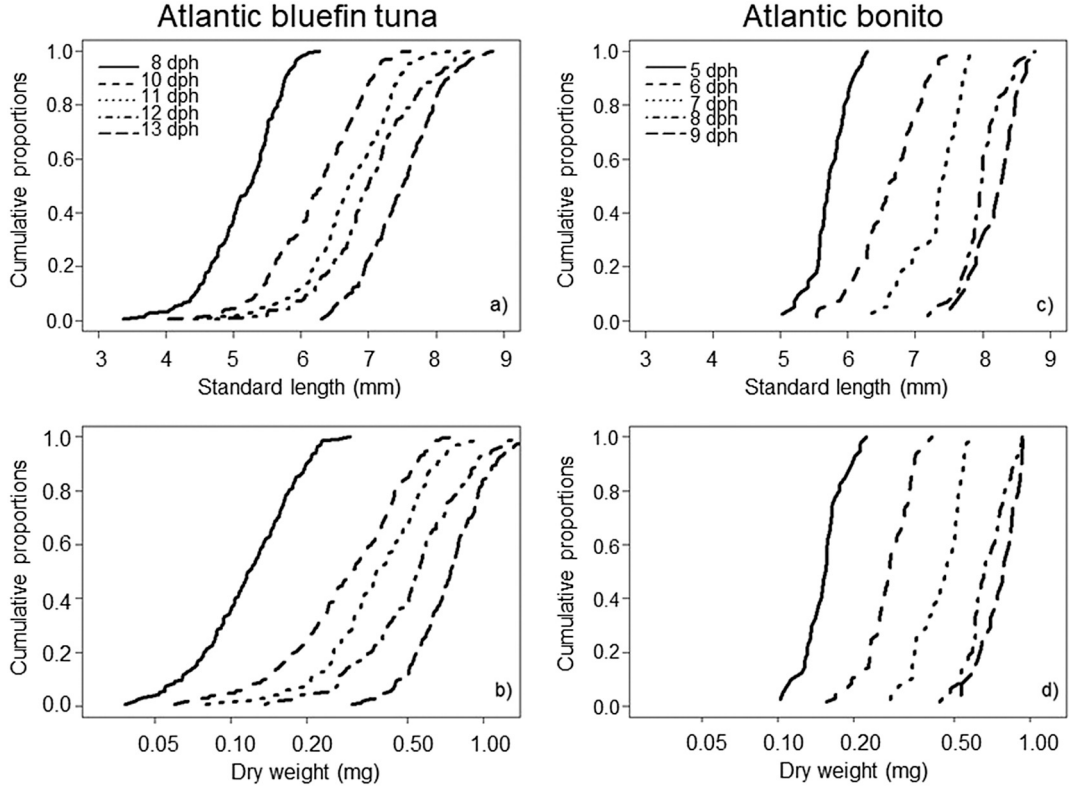


Fig. 1. Cumulative size distribution proportions of standard length (SL, mm) and dry weight (DW, mg). a) Atlantic bluefin tuna SL, b) Atlantic bluefin tuna DW, c) Atlantic bonito SL, and d) Atlantic bonito DW. Different experimental samplings days are shown with different cumulative line type. Note: The x-axis is log transformed for DW in panels b) and d).

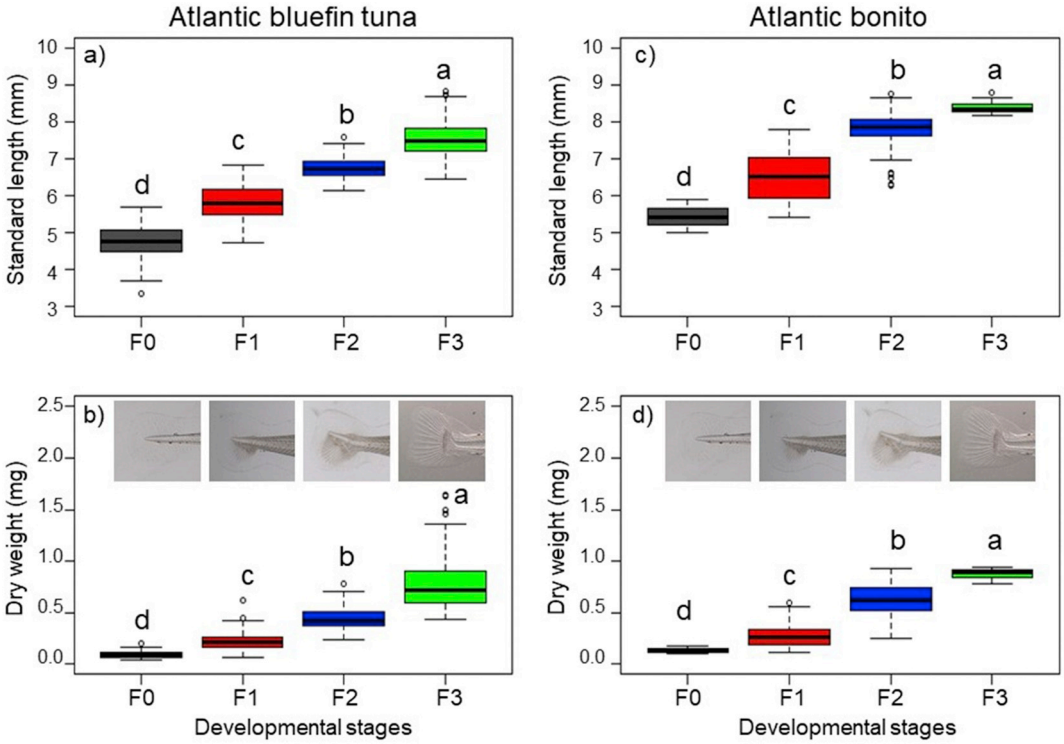


Fig. 2. Boxplots for size rank of standard length and dry weight of each classified developmental stage in Atlantic bluefin tuna and Atlantic bonito larvae. Different letters indicate significant different means among stages (ANOVA, Tukey HSD, $p < .05$).

RNA fluorescence as suggested by Caldarone et al. (2006) assuming for DNA, a ratio of 2.4 RNA content (μg fish^{-1}).

Nucleic acids fluorescence was determined fluorometrically with a Perking-Elmer LS-5 (excitation: 327 nm and emission: 614 nm) by adding 200 μL Ethidium Bromide buffer solution (0.1 mg mL^{-1}). DNA and RNA content were estimated by means of calibrated standards curves of calf thymus DNA (Sigma-Aldrich) and baker yeasts RNA (Sigma-Aldric), respectively. All biochemical analyses of larvae reported in this study were completed within 4–5 months after sampling.

2.5. Statistical analyses

All the statistical analyses were carried out using the R statistical software package (version 3.4.3, Development Core Team, 2017). Final survivals and daily mortalities were estimated from the numbers of initial eggs and the number of larvae counted out at the end of the experiment, subtracting the number of larvae sampled on each sampling day. All size data (SL, DW) were analyzed for heterogeneity of variance (Levene's test) and checked for normality with a Kolmogorov–Smirnov test.

Differences in larval sizes (SL, DW) among replicates within each species were tested using one-way ANOVA, and Bonferroni correction was applied to avoid type I error. A two-way ANOVA test was performed in each one of the 5 studied variables (RNA:DNA ratio, RNA:DW ratio (μg mg^{-1}), DNA:DW ratio (μg mg^{-1}), standard length (mm) and dry weight (mg)) with stage and dph as factors. And when significant, Tukey HSD tests were used for post-hoc comparisons. All test results were considered significant at a level of 0.05.

Cumulative size distributions (CSDs) in standard length and dry weight were estimates as described in Folkvord et al. (2009). Assuming static ranking of fish sizes within a cohort is unlikely to change much in the short term, cumulative size distributions were used for visualizing growth variabilities within cohorts over time in a single graph and can also reveal size-dependent mortality among sampling days (Folkvord et al., 2009). Stage developmental cumulative distribution was also estimated in order to obtain a cumulative approach of the duration of the different developmental stages in each species.

The residuals from the nucleic acid ratios RNA:DNA relationship (RNA-DNA residuals) and DNA:DW relationship (DNA-DW residuals), were compared to the dry weight *vs.* dph (size-at-age) relationship residuals (DW-DPH residuals). A significant positive (negative) correlation indicates faster growing larvae have higher (lower) nutritional condition index than slower growing larvae. In order to determine if the nutritional condition can explain the differences of having different developmental stages at a specific size, the residuals of both nutritional conditions were analyzed against the stage *vs.* dry weight relationship residuals (STAGE-DW residuals). Residuals were analyzed using linear regression and ANCOVA analyses were carried out for stage effect.

3. Results

There were no significant differences in the daily larval sizes among tank replicates in both species (ANOVA, *p-adj.* > .05); therefore, replicates were combined for further analyses. Survival rates of ABFT at 13 dph and AB at 9 dph were 4.1 ± 0.2% and 1.1 ± 0.2% respectively. Further, 27.7 ± 2.5% of the ABFT larvae survived from 13 to 20 dph. On average, every day 23.6 ± 6.4% of ABFT larvae died up to 13 dph, and 16.7 ± 1.0% up to 20 dph. In AB, 39.2 ± 1.6% died daily until 9 dph.

Near-parallel CSDs among subsequent sampling days showed similar growth rates of different size-ranked ABFT and AB larvae both in length (Fig. 1a, c) and dry weight (Fig. 1b, d). However, AB larvae showed lower growth rates from 8 to 9 dph than at other age intervals, as indicated by the almost overlapping cumulative curves (Fig. 1c, d).

There was no significant overlap in the larval length and weight among developmental stages both in ABFT and AB (Fig. 2, Table 3, Table 4, ANOVA, Tukey HSD, $p < .05$). ABFT larvae showed the first signs of flexion (stage F2) from 6.1 mm in length and 0.24 mg in weight and completed flexion (stage F3) from 6.5 mm in length and 0.43 mg in weight (Table 3). In AB first flexion (stage F2) larvae were found from 6.3 mm in length and 0.24 mg in weight and completed flexion from

Table 3
Mean (± SD) standard length (SL, mm) and dry weight (DW, mg) measures of each developmental stage (F0, F1, F2 and F3) found of Atlantic bluefin tuna and Atlantic bonito larvae during our experiment. The exact moment the first larva in flexion (F2) and in post-flexion (F3) was seen is documented, and the standard length and dry weight of that larva is show as *first signs*.

Species	Measurements	F0	F1	F2		F3	
		Average	Average	Average	First signs	Average	First signs
Atlantic bluefin tuna	SL (mm)	4.8 ± 0.5	5.8 ± 0.4	6.7 ± 0.3	6.1	7.5 ± 0.5	6.5
	DW (mg)	0.10 ± 0.03	0.22 ± 0.08	0.43 ± 0.10	0.24	0.77 ± 0.24	0.43
Atlantic bonito	SL (mm)	5.4 ± 0.3	6.5 ± 0.6	7.8 ± 0.5	6.3	8.4 ± 0.2	8.2
	DW (mg)	0.13 ± 0.02	0.30 ± 0.10	0.61 ± 0.20	0.24	0.90 ± 0.05	0.80

Table 4
Summary table of the results from the two-way analysis of variance (ANOVA) among the studied variables. In Atlantic bluefin tuna, analyses were done from 8 to 13 dph and in Atlantic bonito from 5 to 9 dph.

	Atlantic bluefin tuna	Atlantic bonito
	Pr (>F)	Pr (>F)
RNA/DNA		
dph	<0.001	<0.001
Stage	<0.001	0.335
RNA/DW (μg mg^{-1})		
dph	0.323	<0.001
Stage	0.023	0.378
DNA/DW (μg mg^{-1})		
dph	<0.001	<0.001
Stage	<0.001	0.099
Standard length (mm)		
dph	<0.001	<0.001
Stage	<0.001	<0.001
Dry weight (mg)		
dph	<0.001	<0.001
Stage	<0.001	<0.001

8.2 mm in length and 0.80 mg in weight (stage F3) (Table 3).

There was an overlap in the age at which different developmental stages were observed (Fig. 3). In ABFT, F0 stage larvae lasted to 11 dph, while in AB F0 larvae lasted only to 6 dph (Fig. 3). The start of F1 stage was not determined but the development of the rays in the last caudal fin was observed until 13 dph in ABFT and 8 dph in AB. Initial flexion (F2) in ABFT was observed from 8 dph and post-flexion from 9 dph (Fig. 3a). In AB, F0 stage finished around 6–7 dph and F1 at 8 dph. First flexion AB larvae started from 5 dph and post-flexion AB larvae from 7 dph (Fig. 3b). In ABFT, at 10 and 11 dph, larvae of all the stages co-existed, while in AB, at day 6 dph the larvae of the first three stages co-existed.

We found an increasing trend in the daily average RNA:DNA ratio with age in ABFT, whereas a steady tendency was observed in AB independent of age (dph) (Fig. 4). In ABFT daily average RNA:DNA ratio was related to the DNA:DW ratio, since RNA:DW ratio remained steady during the experimental period (Fig. 4a). The daily average DNA:DW ratio decreased with age and developmental stage in ABFT (Fig. 4b), resulting in an increase of the RNA:DNA ratio with age and developmental stage (Fig. 4c, Table 4, ANOVA, Tukey HSD, $p < .05$). In AB, RNA:DNA, RNA:DW and DNA:DW ratios varies with larval age caused by the high variability found in the first sampling days (Fig. 4d, f, Table 4, ANOVA, Tukey HSD, $p < .05$) although they did not vary with developmental stage (Fig. 4d, f, Table 4, ANOVA, $p > .05$).

There was a positive correlation between larval RNA-DNA residuals and DW-DPH residuals (and a corresponding negative correlation between DNA-DW and DW-DPH residual) in ABFT and AB (Supplementary Fig. 1 a, b, c, ANCOVA, $p < .05$). Bigger sized larvae at a specific age, had higher nutritional condition than small sized larvae regardless the developmental stage (ANCOVA, $p > .05$). However, in ABFT, the DNA:DW ratio showed a significative positive correlation (decrease nutritional condition) within stages F0 and F3 (Fig. 5, ANCOVA, $p < .05$). Bigger sized larvae at a specific age within stages F0 and F3 in ABFT had the lower nutritional condition.

The size effect of the larvae was removed by analyzing the condition ratios residuals against larval STAGE-DW residuals (stage-at-size). In ABFT relatively larger larvae within stages F1 and F2 showed a significative higher nutritional condition with regard the other stages (Supplementary Fig. 1d, e, ANCOVA, $p < .05$). However, in AB no relationship was found analyzing DNA-DW residuals (Supplementary Fig. 1 f, ANCOVA, $p > .05$) whereas RNA-DNA residuals showed that relatively larger larvae within F1 stage had higher nutritional condition than the rest of the larvae of any other stage (Fig. 6, ANCOVA, $p < .05$).

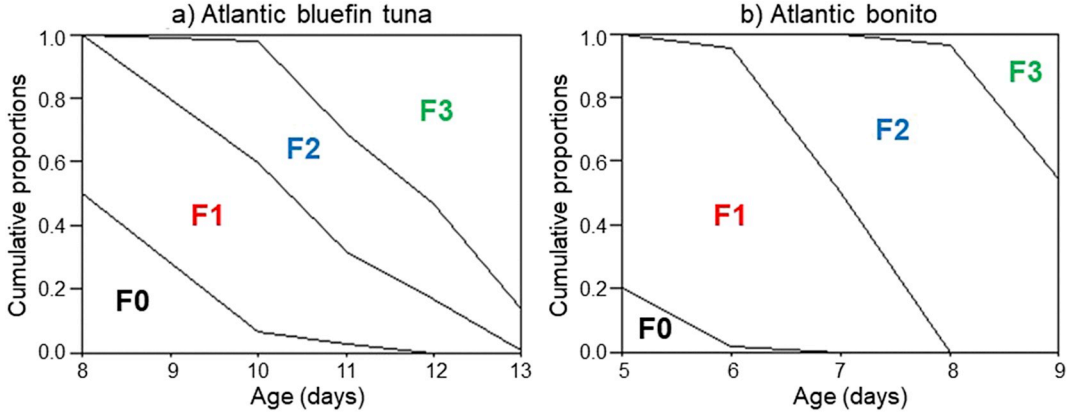


Fig. 3. Cumulative developmental stage proportion distributions showing the duration of the different developmental stages during the a) Atlantic bluefin tuna and b) Atlantic bonito ages. F0: larvae in pre-flexion, F1: larvae with development of the first caudal fin rays, F2: larvae in flexion and F3: larvae in post-flexion.

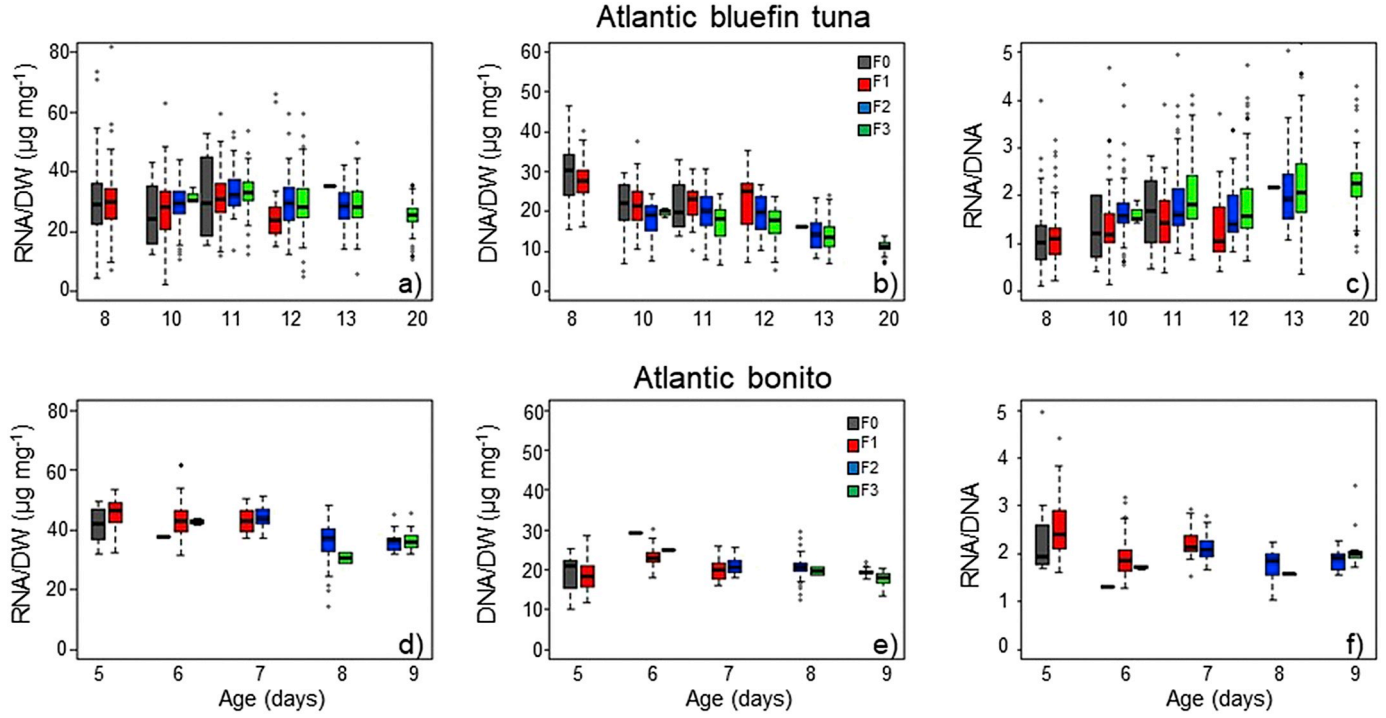


Fig. 4. Boxplots of the daily larval nucleic acid relative measures: RNA:DW (µg mg^{-1}), DNA:DW (µg mg^{-1}) and RNA:DNA ratios in Atlantic bluefin tuna (a–c) and Atlantic bonito (d–f). Different stages are shown in different colors. Atlantic bluefin tuna larvae were sampled from 8 to 20 dph, while Atlantic bonito larvae were sampled from 5 to 9 dph.

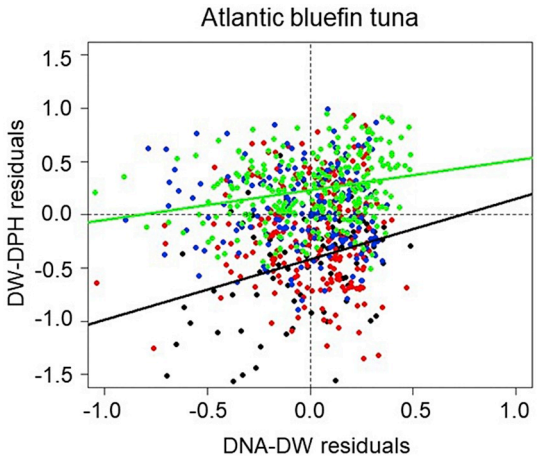


Fig. 5. DW-DPH residuals plotted against DNA-DW residuals in Atlantic bluefin tuna larvae. Different stages are shown with different colors: F0: black, F1: red, F2: blue and F3: green. A significant relationship was found in F0 stage (black line): $y = -0.419 + 0.566 \cdot x$, $R^2 = 0.122$, $p < .01$, $n = 90$ and F3 stage (green line): $y = 0.231 + 0.278 \cdot x$, $R^2 = 0.056$, $p < .01$, $n = 270$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

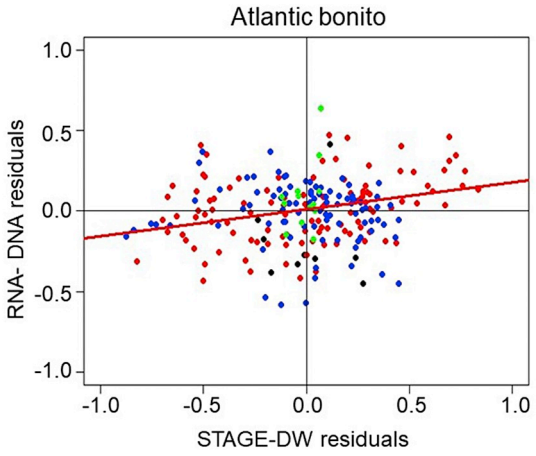


Fig. 6. RNA-DNA residuals plotted against STAGE-DW residuals in Atlantic bonito larvae. Different stages are shown with different colors, F0: black, F1: red, F2: blue and F3: green. A significant relationship was found in F1 stage (black line): $y = 0.012 + 0.167 \cdot x$), $R^2 = 0.102$, $p < .001$, $n = 115$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Understanding what causes developmental variability in fish larvae is fundamental since high mortality during the first days will determine the number of individuals that would reach the juvenile stage, being particularly important in those species where inter-individual variability can lead to cannibalism. The nucleic acid ratios have been widely used to determine the nutritional condition of fish larvae. In this study, we explore if the nutritional condition of the larvae explains differences in the age, size and stage at which development of the notochord occurs during the first days of life in ABFT and AB larvae. We found that the nutritional condition is related with larval growth in both species. At specific size, more developed larvae are related with higher nutritional condition in ABFT, but not in AB.

In our study we have estimated survival rates of 1% and 4%, for AB and ABFT respectively, for the period between hatching and post-flexion, and survival rates of 28% from post-flexion to juvenile in ABFT. Survival rates for laboratory-reared Pacific bluefin tuna and yellowfin have been reported between the time from hatching to juvenile, varying between 0.07% and 3% (Margulies et al., 2007, 2016; Sawada et al., 2005; Tanaka et al., 2018), and from post-flexion to the end of the piscivory phase between 30% and 60% (Blanco et al., 2017; Reglero et al., 2014; Seoka et al., 2008; Tanaka et al., 2014). In AB, survival

from hatching to juvenile varies between 2.9% and 10% (Blanco et al., 2017; De la Gándara et al., 2012; Reglero et al., 2014). These survival data, in accordance with other laboratory data reported, suggest once the larvae reach flexion, the mortality rates decrease significantly. The high mortalities observed during the first days of life in laboratory reared Pacific bluefin tuna or yellowfin have been related to culture techniques that are still in development (Honryo et al., 2016; Nakagawa et al., 2011; Tanaka et al., 2008, 2009), malnutrition (De la Gándara et al., 2012; Margulies et al., 2016; Takebe et al., 2012), adhesion to the surface and to the sinking syndrome (Sawada et al., 2005; Takashi et al., 2006; Tanaka et al., 2009). Besides, rotifers are not the natural preys of tuna larvae from the field which can have an effect in larval survival. Copepods are one of their natural preys and larval survival is known to be improved in comparison with rotifers (Llopiz and Hobday, 2015; Ortega, 2015).

The completion of the head for feeding and respiratory functions, the tail for cruising and escape reactions, and the full development of the intestine appears to be given priority during the first days of life rather than growth in total body length (Osse and Van den Boogaart, 1995). However, as seen from the successive CSDs in larval size, we found similar daily specific growth rates in standard length and dry weight in ABFT and AB with no size-dependent mortality event. The changes in notochord flexion are accompanied by the development of fin rays, changes in body shape, locomotive ability, and feeding techniques (Kendall et al., 1984). Flexion can be used as a proxy for the development of other non-visible changes, such as the appearance of the first gastric glands, the complete development of the stomach with the first pyloric caeca and the development of pharyngeal teeth (Yúfera et al., 2014). Size instead of age is a good proxy for morphological development, since, as we found, there is an overlap in the age at which different developmental stages were observed, while there is no strong overlap in the larval length and weight among developmental stages. The vast majority of the studies regarding tuna larval development are mainly focused on the development of digestive physiology (e.g. Buentello et al., 2011; Miyashita et al., 1998), organogenesis (e.g. Fujimoto et al., 2008; Yúfera et al., 2014) and the development of morphological structures (e.g. Miyashita et al., 2001). Our results from the laboratory indicate first flexion signs from 6.1 mm and first post-flexion signs from 6.5 mm, similar to those reported at 5–5.7 mm and 7–7.4 mm in Pacific bluefin tuna at temperatures of 24.5–27.7 °C and 25 °C, respectively (Kaji et al., 1996; Miyashita et al., 2001).

By the boxplot analyses we found that daily average RNA:DNA ratio during the first days (8–13 dph) varies with the age and development in ABFT larvae. The increasing trend values is correlated with the decreasing DNA:DW ratio and constant RNA:DW ratio with age and development. Same trends have been obtained during the ontogenetic development of other non scombrid species (Bergeron, 1997; Malzahn et al., 2003). The decreasing trend in the DNA:DW ratio suggests a switch in the growth mechanisms of the larval cells from a higher proportion of hyperplasia to a higher proportion of hypertrophy (Buckley et al., 1999; Malzahn et al., 2003). Hyperplastic growth occurring by proliferation of new cells is characterized by mitotic activity, whereas hypertrophy is the enlargement of the existing cells (Weatherley et al., 1988). A decrease in the DNA:DW ratio is achieved by a higher increment in the body weight of the larvae (DW) in relation with the amount of DNA present (genetic material), suggesting a switch to cell enlargement (hypertrophy). Therefore, our results suggest that once the larvae have completed the post-flexion stage and started piscivory, growth by cell enlargement dominates, a trend that has been observed in the juvenile stage of Pacific bluefin tuna (Tanaka et al., 2007). Evident hypertrophy from histochemical analyses has been documented from 29 dph in Pacific bluefin tuna juveniles, indicating hyperplasia persisted over a long period of time (Roy et al., 2012, 2014). In AB larvae, daily average DNA:DW ratios remained unchanged with development but not with the age (probably caused by the high variability in some days) which may be explained by an earlier combination of hyperplasia and hypertrophy in AB compared to ABFT larvae. The dynamic of hyperplasia and hypertrophy has been seen to determine the ultimate somatic size of the fish. In small adult size species hyperplasia ceased early and most of the growth is attributed to hypertrophy, whereas in those attaining large adult sizes hyperplasia continued for a long time in the development (Weatherley et al., 1988). AB adult sizes are much smaller than those of ABFT, which may explain the possible difference in cell growth mechanisms.

The decreased tendency of average DNA:DW ratio with age (and size) might be due to artifacts during the homogenization and sub-sampling protocol. Larger larvae may be more difficult to homogenize by sonication, and thus, they might not be completely digested by the time of the sub-sampling of the homogenate. In this case, the supernatant sub-sample in larger larval homogenates might contain less nucleic acid than the supernatant from smaller, more easily digested larvae and the sub-sample might not represent the original larval dry weight. Moreover, it is possible that some tissue types are digested more easily than others and this would also get a bias sample of some tissue types in greater proportion than others, with potential differences in nucleic acid ratio in those different tissues (Olivar et al., 2009). The higher average RNA:DNA ratio values in ABFT than AB may be explained by the high proportion of the head tissue into the biochemical analyses, resulting in a decrease in the total RNA:DNA ratio. The RNA:DNA ratio in the head of several species is lower than in the muscles due to higher DNA:DW ratio derived from the presence of a larger number of small cells in the head (Olivar et al., 2009). Olivar et al. (2009) also suggested that the lower RNA:DNA ratio in the head than in muscle could indicate lower growth rates than muscle tissues related to lower protein synthesis. AB larvae are characterized for having a very big head, representing more than a third of the body size, until reaching the juvenile stage, whereas ABFT's head is also bigger than in other larval fish species, but proportionally smaller than in AB (Rodríguez et al., 2017).

A size effect (positive correlation) was found by the residual analysis of RNA:DNA ratio and larval size (or growth) at specific age in both species, where bigger larvae had a higher nutritional condition, and also suggesting good larval condition in ABFT and AB larvae during the five days or more before sampling. However, the cell condition ratio, DNA:DW residuals in ABFT showed a decrease in the cell condition within the developmental stages F0 (pre-flexion) and F3 (post-flexion), not in AB. The biochemical measures of cell size (DNA:DW ratio), rather than the protein synthesis capacity (RNA:DNA ratio), was a better indicator of the condition of the different flexion stages in ABFT larvae (Bergeron, 1997). In ABFT larvae, where more larvae were sampled, the RNA:DNA ratio measurements were highly variable, stemming from the high variability obtained in the RNA. The RNA:DNA ratio might not be as strongly related to larval feeding condition as it is the DNA:DW ratio which is considered more stable. It may also be more sensitive to the nutritional status of the larvae because cell weight is decreasing while the amount of DNA is kept constant if feeding decreases (Bergeron, 1997). Besides, the DNA:DW ratio may be more related to the larval stage of fishes while the RNA:DNA ratio seems to be more related to feeding condition during the late larval of juvenile stage as was pointed out in Bergeron (1997).

Since there is a size effect on the nutritional condition of the larvae, the nucleic acid ratios against the developmental stage at a specific size were used to determine if the nutritional condition is responsible for the different developmental individuals at the same size. In ABFT, in general, we found that at a given size more developed larvae are related with higher nutritional condition and within each developmental stage, nutritional condition of the relatively larger larvae in flexion stages (F1 and F2) was higher than those in pre-flexion (F0) and post-flexion (F3). Fast growth during the first days of life (F0 stage) along with a possible suboptimal feeding shortly after yolk absorption, may be responsible of the decrease in the nutritional condition of the larvae. We saw the first signs of post-flexion at 10 dph while the larvae offered as prey was first

offered to the tanks at 13 dph. The timing to switch to piscivory in ABFT is known to determine further larval growth and survival and a delay of 4 or 8 days can increase mortalities (Reglero et al., 2014). The delay in prey switch in our experiments may have affected the nutritional state of those larvae already in post-flexion (F3) at 10 dph (Takebe et al., 2012). This might be apparent in the nucleic acids (fast response) but not yet in the larval weight. The suboptimal feeding of larvae in post-flexion might be hiding an increase in RNA:DW ratio once the notochord flexion is completely finished. The increased energy demands due to the active swimming of the post-flexion larvae might exceed the reduced energy gain during suboptimal feeding decreasing larval condition (Billerbeck et al., 2001; Faria et al., 2011; Illing et al., 2018; Lankford Jr et al., 2001; Moyano et al., 2018). Silva et al. (2015) in plaice larvae (*Pleuronectes platessa*) concluded that larvae with lower DNA:DW ratio had better swimming abilities, however they did not find a relationship with the RNA:DNA ratio. In AB at a given size, relatively larger individuals had higher nutritional condition only within stage F1. The decrease in growth seen from 8 to 9 dph, might show how the delay in prey switch in our experiments might have affected the nutritional state of those larvae at more advanced stages.

Our results clearly indicate that when parameters such as diet and temperature do not vary, cell growth mechanisms are species-specific. Under culture, *ad libitum* conditions, as seen in our results, feeding conditions obtained from nucleic acid derived indices might not be enough to explain developmental differences in ABFT and AB larvae. The explanation of growth differences and developmental rates in those fast-growing fish larval species might be difficult to identify since short time intervals separate different stages and differences in growth strategies between individuals (Juan-Jordá et al., 2013). The nutritional condition measured by the nucleic acid derived indices RNA:DNA and DNA:DW ratios are only two measures of biomolecular condition and other condition analyses such as lipid levels or histological indices along with other aspects such as energy consumption (e.g. swimming, specific dynamic action) should be investigated in order to better understand larval species-specific strategies. As seen in other pelagic larval species, the physiological response to nutrition level can be translated to affect the viability of the larvae after handling (cod, Øie et al., 2017), regulate swimming and metabolic rates (herring, Illing et al., 2018) and has a potential long-term effect in growth and condition when reaching the juvenile stage (herring, Folkvord et al., 2018).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2019.151182.

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